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REVIEW ARTICLE

The mechanism of vesicle formation

Danilo D. LASIC*
Department of Physics, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada

INTRODUCTION

Phospholipid molecules exhibit amphiphilic properties and therefore they aggregate either in their crystalline state or in polar solvents into ordered structures with typical lyotropic liquid crystalline symmetries (de Gennes, 1974). At high lipid concentrations in water these are predominantly lamellar phases (Luzzati, 1968), while in aqueous solutions phospholipid molecules normally form self-closed spherical or oval structures where one or several phospholipid bilayers entrap(s) part of the solvent in its/their interior (Bangham & Horne, 1964; Papahadjopoulos, 1978).

In the case of one bilayer separating the internal and external solvent these structures are called, with respect to their size, small or large unilamellar vesicles (SUV and LUV, respectively) while the term multilamellar vesicles (MLV) is used in the case of many bilayers entrapping some of the solvent. The terms giant vesicle (GV) and large or small oligolamellar vesicles (LOV, SOV) are also used for very large vesicles and structures where several bilayers surround the entrapped solvent. Sometimes, especially in the case of technological applications, the term liposome is a homonym for SUV, LUV and MLV while in the older literature terms liposome and MLV are often synonyms.

Vesicles are very important in many different areas of science and technology. In basic research they serve as models for cell membranes and their fusion, transport studies and investigations of membrane proteins that can be reconstituted in vesicles (i.e. 'proteoliposomes'). They also serve as delivery vehicles for drugs, genetic material, enzymes and other (macro)molecules into living cells and through other hydrophobic barriers in pharmacology, medicine, genetic engineering, cosmetic industry and food industry (Gregoriadis, 1984). They are also used as a support for semiconductor particles, in applications such as the photoconversion of solar energy (Zhago *et al.*, 1988).

Despite the extensive use and diverse applications of vesicles the mechanism of their formation is not yet well understood. Several factors contribute to our scarce knowledge of this process. Physical and chemical methods which would enable us to study short-lived intermediate structures in the vesicle formation process are not available. The inherent thermodynamic instability of these transition structures makes analysis by spectroscopic, thermodynamic, hydrodynamic, diffraction and microscopic techniques very difficult.

Quite often the results are also nonreproducible and the physical characteristics of vesicles produced (size distribution, lamellarity, stability...) depend on the history of the sample and precursor phases, the path of obtaining/treating these phases and preparation procedure. The perplexity of the situation is compounded by the facts that vesicles can be produced by many different methods which do not seem to have anything in common and that most of the researchers were interested mainly in the development of recipes for well-characterized, reproducible vesicle preparations and not in the physical and chemical origin of parameters involved in the preparation procedures.

In the beginning of this decade, however, a model of vesicle formation by a detergent-depletion technique, one of several preparation methods, was proposed (Lasic, 1982*a*,*b*). It was based on energy considerations developed earlier (Franck, 1958; Ferguson & Brown, 1968; Helfrich, 1974) and which predicted a disk-like phospholipid micelle as an intermediate structure in the vesicle formation process. Such structures were already described in the literature in the studies of bile salt/lecithin micelles (Small *et al.*, 1969; Mazer *et al.*, 1980). Later this model was generalized to encompass the other preparation techniques which, as a consequence, could be at least qualitatively understood (Lasic, 1983, 1985, 1987). Several research groups also tried to prove this model experimentally (Schurtenberger *et al.*, 1984; Fromherz & Ruppel, 1985; Almog *et al.*, 1986; Cornell *et al.*, 1986; Wrigglesworth *et al.*, 1987). The aim of this article is to review, after a brief introduction of vesicle preparation methods, the present state of the understanding of the vesicle formation. The existing methods will be classified into a logical, unifying scheme. For more pictorial presentation and easier understanding, several schematic drawings are included.

It is hoped that this review, with the addition of some speculative thoughts, will inspire some new experiments, explanations and generalizations in this field where on one side so many details are known and on the other so little is understood.

PREPARATION METHODS

MLVs form spontaneously when dry phospholipid films swell in excess water or buffer (Bangham & Horne, 1964; Bangham *et al.*, 1965). LUVs and SUVs, however, have higher free energies and some energy must be dissipated into the system in order to produce them. Exceptions are charged phospholipids which also form some uni- and oligo-lamellar structures (Hauser *et al.*, 1985) and some double-chained surfactants which form spontaneously stable vesicles (Talmon *et al.*, 1983).

Fig. 1 shows an approximate energy diagram of different aggregates of the uncharged phospholipid at

Abbreviations used: MLV, multilamellar vesicle; LOV, large oligolamellar vesicle: SOV, small oligolamellar vesicle; LUV, large unilamellar vesicle; SUV, small unilamellar vesicle; BPF, bilayered phospholipid flake; GV, giant vesicle.

* Present address and address for reprint requests: Liposome Technology Inc., 1050 Hamilton Court, Menlo Park, CA 94086, U.S.A.

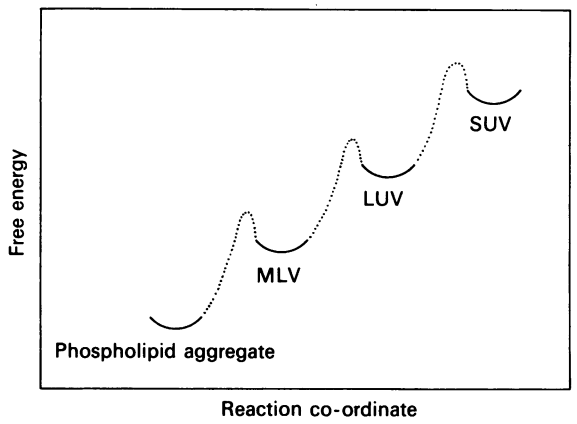


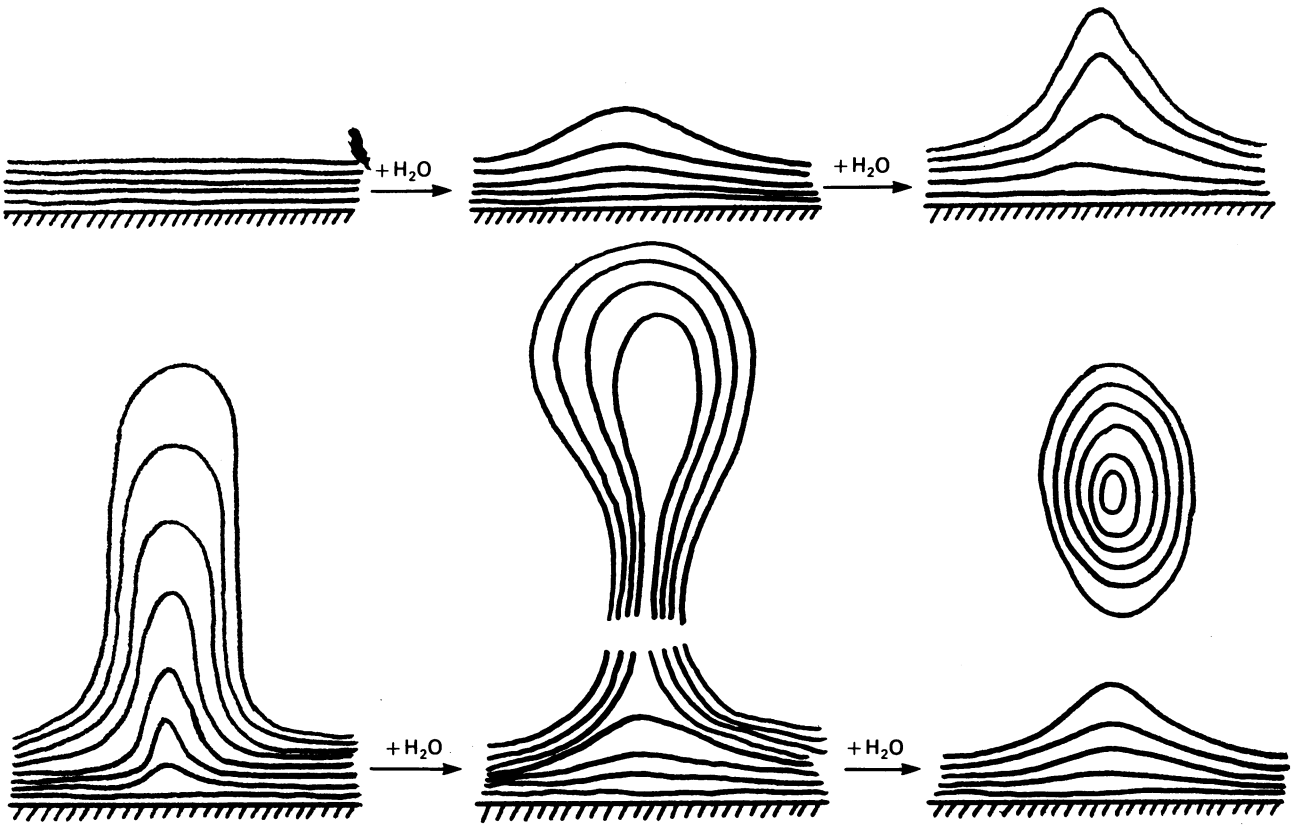
Fig. 1. An energy diagram of different phospholipid aggregates

The energy minima of different structures are positioned only very approximately. In the case of low values of elastic curvature modulus (k_c) the energy difference between LUV and SUV decreases and in the case of extremely low k_c it may, due to entropy contribution, even reverse.

low concentrations in water. The state with the lowest free energy is a hydrated precipitate. MLVs have slightly higher energies and to achieve LUVs and SUVs even more energy must be dissipated into the system. Normally SUVs are stable up to several weeks after which time they aggregate and fuse into LUVs or MLVs. After several days/weeks (depending on concentration, purity, temperature, ionic strength...), a solution of LUVs transforms into MLVs. As time progresses a one-component dispersion of MLV demixes into a two-phase system in which phospholipid-rich/water-poor and phospholipid-poor/water-rich phases coexist.

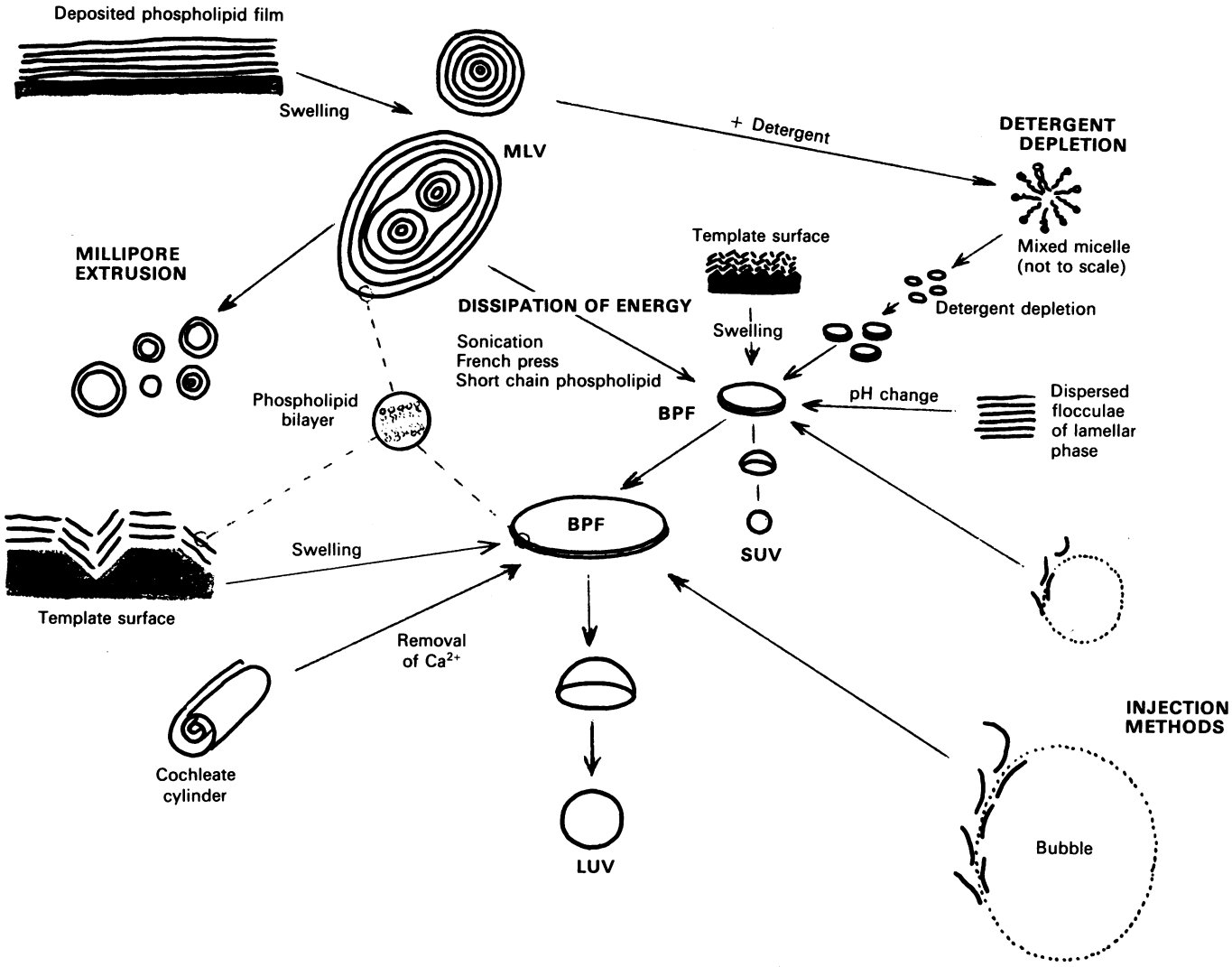
The transformation of SUVs/LUVs into MLVs or hydrated phospholipid aggregates upon freeze–thawing or dehydration–rehydration cycles can be envisaged from Fig. 1. It is interesting to note, however, that addition of some sugars prevents this phase transformation and upon thawing or rehydration SUVs/LUVs are reformed (Crowe *et al.*, 1985). This phenomenon is probably due to the specific interaction of sugars with polar heads and can be very useful for the long-term storage of vesicles (Strauss & Hauser, 1986; Crowe *et al.*, 1987).

In this article the major preparation methods will be presented only very briefly. For the additional details an interested reader is referred to reviews by Szoka & Papahadjopoulos (1980) or Hauser (1982) and original references therein. However, in addition, and in contrast with other reviews, in this article a new section, describing spontaneous vesiculation which also can produce SUVs and LUVs, is added. Besides the methods described in this section there exist many other preparation methods. However, they are for the most part combinations and variations of the methods discussed below. In fact, most of the preparation procedures were introduced earlier,



Scheme 1. A possible schematic representation of the formation of MLV upon the hydration of the dry phospholipid film

Lines indicate phospholipid bilayers. The swollen tubular fibrils can be very long (up to 100 μm) and occasionally they have undulating structure which in the optical microscope looks like a string of beads. The geometry of these structures may be correlated to the initial ratio of molecules in the outer and inner monolayer.



Scheme 2. A schematic drawing indicating the formation of vesicles

The cartoon shows how bilayered phospholipid fragments (BPF) can be formed using different vesicle preparation methods. BPF can be grown by detergent depletion, phospholipid precipitation or they can be formed from pre-existing bilayers. They can be prepared also directly by using template surfaces. This scheme shows that the BPF is an intermediate structure in the vesicle formation process by different preparation procedures. The thermodynamic instability at the edges of the BPF causes bending and when the BPF closes upon itself a vesicle is formed.

although their results were not necessarily understood properly (Saunders, 1953; Robinson, 1960; Saunders *et al.*, 1962). After the schematic presentation of the possible mechanism of the MLV formation (Scheme 1), most of the vesicle preparation techniques are graphically illustrated in Scheme 2.

Multilamellar vesicles

Originally MLVs were prepared by the swelling of dry phospholipid films deposited in a round-bottomed flask in excess water under gentle or vigorous shaking (Bangham & Horne, 1964; Bangham *et al.*, 1965). Depending on the input energy [swirling, shaking, vortexing, nature of phospholipid(s), ionic strength, nature of ions, concentration, purity ...], MLVs of different size distributions are formed (Scheme 1). In the case of gentle swirling their sizes, lamellarities and size distributions have a very broad spread with sizes varying from 0.1 to several dozens of μm. A relatively well defined size distribution (1–4 μm) is obtained if the energy input (such as vigorous vortex-mixing for 10 min) as well as the composition and concentration are carefully controlled.

MLVs with narrower size distributions can be formed by the solvent spherule method (Chowhan *et al.*, 1972; Kim *et al.*, 1985) whereby uniform spherules of phospholipid and organic phase in oil-in-water emulsion transform into MLVs upon removal of the organic phase. The reverse phase evaporation method for the preparation of LUVs (see below) also can be applied to the formation of uniform MLVs. To prepare MLVs instead of LUVs only larger concentration of phospholipid(s) have to be used (Pidgeon & McNeely, 1987). The advantage of the methods that start from reverse phases (i.e. water-in-oil emulsions) is the much larger efficiency of entrapment of water-soluble substances. This is the consequence of the fact that during the formation of MLVs or LUVs water is being forced to be entrapped by phospholipid which is distributed at oil/

water interfaces in the emulsion. By slight changes in the preparation procedure large vesicles with entrapped smaller vesicles (multivesicular vesicles) can also be prepared (Talsma *et al.*, 1987, and references therein).

MLVs can be prepared also from preformed SUVs or LUVs either by controlled fusion, freeze–thawing or by a dehydration–rehydration cycle. Evidently, the last method again increases encapsulation efficiency of water-soluble molecules. Better-defined size distributions are achieved also by extrusion of MLVs through polycarbonate filters (Olson *et al.*, 1979). However, the MLVs formed may not have lamellae.

Particular phospholipids, or their mixtures, can form MLVs or LOVs also by dehydrating dry phospholipid powder(s) in water/buffer during vigorous agitation (in a high shear mixing apparatus, for example) or by hydrating dry phospholipid spherules produced by spray drying and lyophilization of phospholipid(s) in an organic solvent.

Small unilamellar vesicles

These vesicles, with sizes ranging from 20 to 100 nm, can be prepared by many different techniques. The most widely used technique involves sonication of MLVs (Saunders *et al.*, 1962; Papahadjopoulos & Watkins, 1967; Huang, 1969). They can be produced also by forcing a suspension of MLVs through a French press (Barenholz *et al.*, 1979). The most useful method for reconstitution of membrane proteins is the detergent depletion technique. Normally MLVs are dissolved with detergent which is later removed by dilution, dialysis, chromatography, adsorption, ultrafiltration or centrifugation, and SUVs are formed (Kagawa & Racker, 1971; Brunner *et al.*, 1976).

Large unilamellar vesicles

These vesicles, ranging from 0.1 to 1 μm, can be prepared by the detergent-removal technique. However, different detergents and detergent depletion rates (see below) are used (Nozaki *et al.*, 1982, and references therein). Injection methods, where phospholipid(s) dissolved in an organic solvent are injected into warm water, also yield LUVs (Deamer & Bangham, 1976) although SUVs can form as well (Batzri & Korn, 1973). A mixture of SUVs and LUVs is produced when a suspension of charged phospholipid(s) [or mixture of neutral phospholipid(s) with at least several % (w/w) of charged amphiphile] are subjected to a cyclic change of pH (Hauser & Gains, 1982). A dispersion of charged phospholipid in buffer and in the presence of Ca^{2+} (i.e. cochleate cylinders) forms LUVs upon removal of Ca^{2+} ions (Papahadjopoulos, 1978).

A very suitable method, especially for the encapsulation of hydrophilic molecules, is the reverse phase evaporation method (REV) where LUVs are formed by depletion of the organic phase from water-in-oil emulsions of phospholipid(s) and water in an organic phase (Szoka & Papahadjopoulos, 1978). LUVs and/or LOVs at very high concentrations (up to 50 wt%) can also be prepared by ethanol/Freon injection of very concentrated phospholipid solutions into aqueous media. The organic solvent is removed at the same rate as it is introduced leaving a viscous liposome paste. Such methods can be operated as a continuous process (not in batches) and are easy to scale up to commercial quantities (Martin & West, 1988).

Giant vesicles

Vesicles with diameters up to 50 μm were prepared by very careful shakeless swelling of very uniform thin phospholipid films in pure warm water (Reeves & Dowben, 1970). With proper adjustment of experimental conditions (careful crystallization of phospholipid film and its swelling, temperature, addition of stain) it is probably also possible to prepare giant uni- or oligolamellar vesicles visible to the naked eye.

SPONTANEOUS VESICULATION

In contrast to finite swelling behaviour (i.e. equilibrium distance between hydrated bilayers in excess water is finite, usually below ~ 5 nm) of uncharged phospholipids, charged phospholipids or their mixtures exhibit infinite swelling behaviour upon hydration. The surfaces of bilayers ionize and they repel each other with equilibrium distance between lamellae approaching infinity (Krzywicki *et al.*, 1969). Deposited films of charged phospholipids yield heterogeneous populations of LOVs, MLVs (with much larger separations between lamellae) and also LUVs and some SUVs upon swelling in water (Hauser *et al.*, 1985). Similar effects were achieved also by using an electric field during swelling (Angelova & Dimitrov, 1986). An underlying theory was proposed (Lipowsky & Leibler, 1986).

It was shown that SUV can coexist with other phase(s) in certain regions of the phase diagram of the egg yolk lecithin/ionic detergent/water system (Rydhag *et al.*, 1982), while a peculiar class of synthetic surfactants with charged polar heads spontaneously arrange into thermodynamically stable unilamellar vesicles in water (Talmon *et al.*, 1983).

The infinite swelling of charged phospholipids is described by Hauser (Hauser, 1984; Hauser & Lasic, 1985). This effect was used in conjunction with the topography of the surface on which the phospholipid film was deposited to produce homogeneous preparations of SUVs and LUVs by Lasic (Lasic, 1988; Lasic *et al.*, 1987, 1988). Vesicles were formed spontaneously also by mixing long chain phospholipid with short chain phospholipid, or lysolecithin, either already in the organic phase or adding short chain phospholipid to a dispersion of MLVs (Gabriel & Roberts, 1984; Hauser, 1987).

MECHANISM OF VESICLE FORMATION

In this section I shall first qualitatively describe the mechanism of the formation of MLVs. Then a mechanism of SUV and LUV formation by the detergent-depletion technique will be described and later it will be generalized to other preparation methods. At the end all the vesicle preparation methods will be unified into a logical scheme and some other possible models will be discussed.

Multilamellar vesicles

Although the swelling of dry phospholipid films in water, i.e. growth of myelin figures, has been known for a long time (Lehman, 1911) the detailed study of MLV formation has not yet been reported. However, the observation of this process in the phase contrast optical microscope can yield some geometrical clues. When noncharged phospholipids are hydrated the myelin figures grow normally in the form of tubular fibrils which elongate (Harbich & Helfrich, 1984). One can speculate

that by adding water to the dry phospholipid film the outer monolayer hydrates more than the inner ones. The convex bumps (blisters) are formed because the surface area of polar heads increases with the increasing hydration (Saupe, 1975). Water penetrates in between the bilayers as well as through such bumps. The hydration reaction reduces the energy of the system which causes the system to increase its specific surface area. Bilayers grow from such blisters into tubular fibrils, greatly increasing the contact area with water. Hydrating bilayers are sliding into fibrillar myelin figures, in order to expose the polar heads to water maximally. During this transformation the bilayers stabilize into their equilibrium distance which is a compromise between the repulsive undulation/steric and attractive van der Waals forces. Some possible crystal or packing defects at one side/point of the primary blister can induce torque such that fibrils grow in helices which are often observed. Upon agitation these tubes detach and immediately seal off their exposed edges and form MLVs. It is easy to see that the intensity of the agitation (from intense homogenization, vortex-mixing and stirring to only thermal excitations) influences the size (distribution) of the MLVs formed. They are not necessarily spherical but rather oval in shape because of the nature of their formation (Scheme 1). In some spots, depending on the local crystallization defects, bunches of lamellae peel off and they close to form MLVs. Normally such a bunch of flakes blows up in its middle, slides with the edges attached to the lower bilayer, disconnects from the surface and finally the lamellae close upon themselves. With time nonspherical structures are probably slowly transforming into spherical ones where their curvature energy is minimal and the entrapped volume maximal. This is probably achieved via a directional flip-flop of phospholipid molecules because the number of molecules in the outside monolayer, which depends on the events occurring during MLV formation and also dictates its shape, may be much larger than about one-half of all phospholipid molecules in the structure.

Mechanism of vesicle formation by detergent depletion

In contrast with most of the other vesicle formation methods where vesiculation occurs almost instantly, the intermediate structures have longer stability within this preparation procedure. Therefore it was the first method for which a model of vesicle formation was proposed (Lasic, 1982*a*,*b*) and for which an experimental search for the mechanism and intermediate structure was performed (Schurtenberger *et al.*, 1984; Almog *et al.*, 1986; Cornell *et al.*, 1986). Later it will be shown that the same mechanism can be applied to the other preparation techniques as well (Lasic, 1987).

The starting solution for this preparation techniques is a mixed micellar solution of phospholipid dissolved in detergent micelles (it can be prepared by dissolving MLVs with detergent or directly by simultaneous precipitation of both lipids from organic phase). Upon removing detergent from small detergent/phospholipid mixed micelles they grow by fusion (the only other way to grow would be by directional phospholipid exchange). The energetically unfavourable boundary interaction (Lasic, 1982*b*) at the (shielded) edges of micelles of the system is proportional to the total circumference (S) of disk-like micelles, since:

$$E_b = \gamma_{eff.} \cdot 2\pi\Sigma_i r_i \qquad (1)$$

where $\gamma_{eff.}$ is the effective interaction at the edges and i is summed over all micelles with radii r_i. The system reduces total E_b by fusion because at constant surface area of phospholipid molecules the total circumference, $\Sigma_i S_i$, decreases ($\Sigma_i S_i > \Sigma_{i'} S_{i'}$, $i' < i$ at constant number of phospholipid molecules). However, when there are not enough detergent molecules to shield the exposed hydrocarbon core from the polar environment (increase of $\gamma_{eff.}$) the micelles start to bend, further reducing $\Sigma_{i'} S_{i'}$, until they eliminate this unfavourable exposure by closing upon themselves. Of course the system pays for this decrease of boundary interaction upon bending (perimeter of disk with radius r decreases as $r\sqrt{(1-r^2/4R^2)}$ with R being the curvature radius) by increasing its curvature energy E_c. Below a certain size of micelles curving would require stretching the area of polar heads ($\tilde{a}$) in the outer monolayer and compressing $\tilde{a}$ in the inner one. This contribution to the energy, the inelastic curvature energy which is inversely proportional to r can be, in analogy with the stretching of smectic liquid crystals, defined as:

$$E_h = k_{cc}\left(\frac{\Delta\tilde{a}}{\tilde{a}}\right)^2 \cdot \pi r^2 \qquad (2)$$

per micelle with k_{cc} being the stretching modulus of membrane (Helfrich, 1973). The factor $\frac{1}{2}$ is in the first approximation omitted assuming that energy contributions from stretching and compressing $\tilde{a}$ are equal. For small values of r, large changes in $\Delta\tilde{a}$ would have been required and it follows that $E_h \gg E_b$. The value of E_h is rapidly decreasing with increasing r and above a certain size of mixed micelle (r_c) we can assume that the bending is elastic (i.e. $\Delta\tilde{a} = 0 \rightarrow E_h = 0$) and Hooke's law can be applied (Lasic, 1982*b*). This assumption could be easily omitted by expressing $\Delta\tilde{a} = \Delta\tilde{a}(r)$ quantitatively and defining curvature energy as a sum of a linear term (elastic, E_c, see below) and a quadratic (inelastic, E_h) term which rapidly decreases with increasing r (D. D. Lasic, unpublished work).

The elastic curvature energy is defined as

$$E_c = (2k_c + k_c')\Sigma\pi_i r_i^2/R_i^2 \qquad (3)$$

where the elastic modulus of bending includes splay (k_c) and saddle splay (k_c'). (Franck, 1958; Helfrich, 1974). For disk-like micelles which have no torque and force densities within the bilayer the curvature energy depends only on boundary conditions and we can assume $k_c' = 0$ (Helfrich, 1973). This assumption is probably valid for phospholipid/detergent systems above the gel–liquid crystal phase transition. The total excess energy of a flat ($R = \infty$) or curved mixed micelle (with curvature $1/R$) can therefore be written as:

$$E_e = \gamma_{eff.} \cdot 2\pi r \cdot \left(1 - \frac{r^2}{4R^2}\right)^{\frac{1}{2}} + k_{cc}\left(\frac{\Delta\tilde{a}}{\tilde{a}}\right)^2 \pi r^2 + 2k_c\frac{\pi r^2}{R^2} \qquad (4)$$

Assuming that above a certain size of mixed micelles ($r > r_c$), $\Delta\tilde{a} = 0$ upon bending, therefore the middle term in the equation equals zero, and we can see that when the curving bilayered micelle closes upon itself the first term vanishes ($r = 2R \rightarrow S = 0$), yielding constant E_e per vesicle:

$$E_e = 8\pi \cdot k_c$$

regardless of vesicle radius. This shows that energetically (but not entropically) large vesicles are preferred over small ones and E_e is directly proportional to the number of vesicles n, $E_e = n \cdot 8\pi k_c$ (Israelachvili *et al.*, 1980;

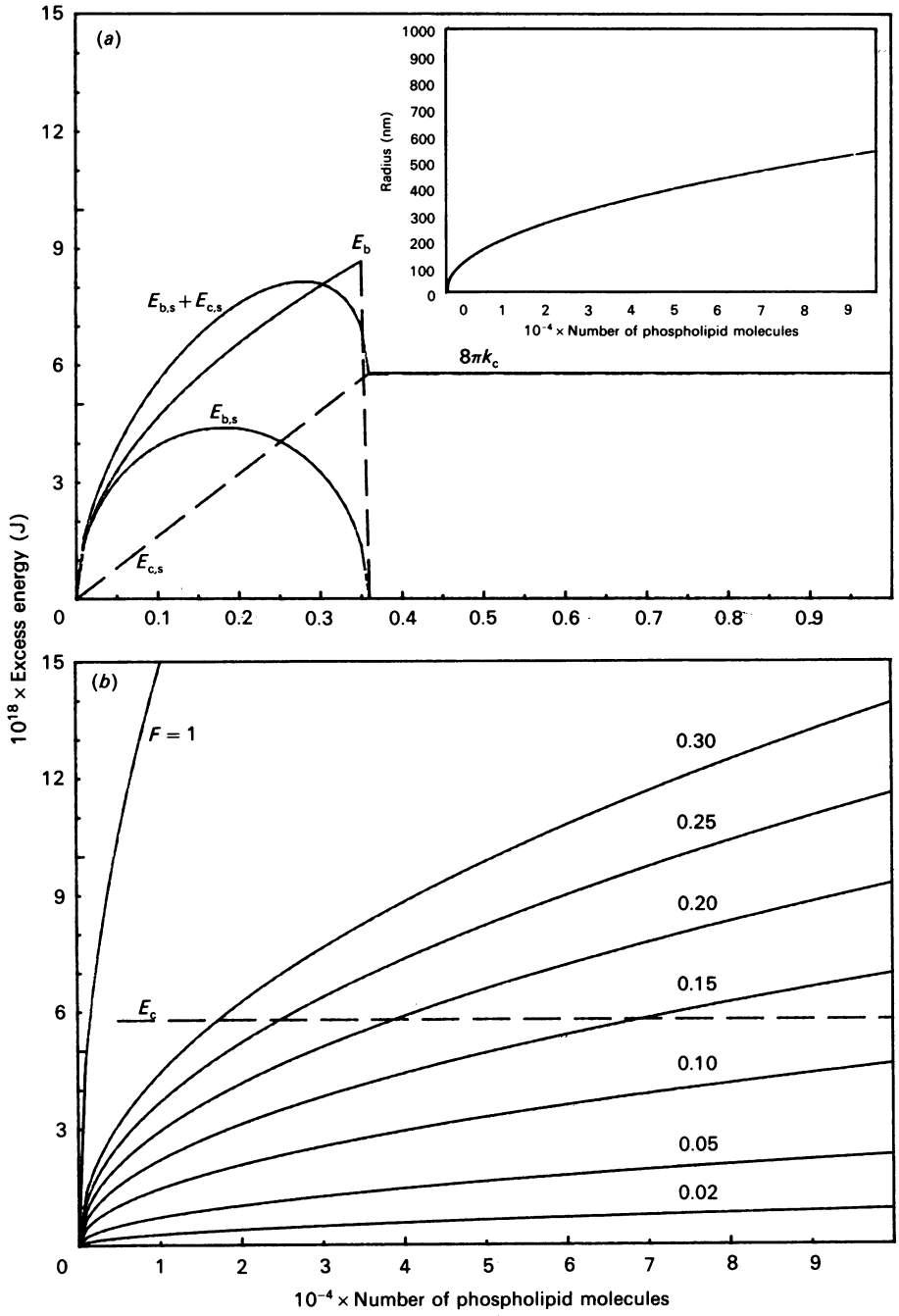


Fig. 2. Formation of SUV and LUV

(*a*) SUV formation: the excess energy (E_e) as a function of the number of phospholipid molecules in the structure. E_b shows the boundary energy of the flat-like micelle, $E_{b,s}$ represents the boundary energy for a curved micelle which grows into the surface of a sphere with $R = 10$ nm, $E_{c,s}$ shows the curvature energy of this curved micelle while the total excess energy of this curved micelle is characterized by $E_{b,s}+E_{c,s}$. From this energy diagram and according to this simplified model of vesicle formation it can be concluded that the growth of a flat-like micelle is energetically favoured up to the last stages of the vesiculation process where curving lowers the total excess energy. When a vesicle is formed $E_b = 0$ and its $E_e = E_c = 8\pi k_c$ regardless of its radius. For this calculation the values $\gamma = 7 \times 10^{-20}$ J/nm, $k_c = 2.3 \times 10^{-19}$ J, $\tilde{a} = 0.7$ nm^2 and $R = 10$ nm were used. The inset shows the number of phospholipid molecules in a vesicle as a function of vesicle radius R. (In this calculation the thickness of the bilayer of 4.5 nm was used and it was assumed that $\tilde{a} = 0.7$ nm^2 in the inner and outer monolayer. (*b*) LUV formation: the excess energy (E_e) as a function of the number of phospholipid molecules (n) in the flat-like mixed micelle. The boundaries of mixed detergent/phospholipid micelles are shielded by the detergent and the value of $\gamma_{eff.}$ is reduced according to $\gamma_{eff.}^{(sh)} = F \cdot \gamma_{eff.}$, where F is the shielding factor with values $0 \leqslant F \leqslant 1$ (Lasic, 1982*b*). The dotted line indicates the excess energy of vesicle after closure [$E_e = E_c = 8\pi k_c \neq f(R)$] while the family of curves shows the E_b of a flat-like shielded micelle containing n phospholipid molecules for different values of F. When $E_b \geqslant E_e$ mixed micelles can overcome the energy barrier for closing ($8\pi k_c$). This energy is provided by thermal energy ($\sim 3.7 \times 10^{-21}$ J/molecule at room temperature) and/or other excitations. The closure is practically irreversible because the energy required to open the closed vesicle and create open edges is at these detergent/phospholipid ratios much larger than $8\pi k_c$.

Lasic, 1985). Different detergents have different shielding abilities due to their size, geometry, amphiphilicity, and values of distribution coefficient between edges/interior of mixed micelles. In addition their critical micelle concentration values dictate the rate of detergent removal, i.e. the rate of vesicle formation. This also can be controlled by experimental procedure (D. D. Lasic & Y. Nozaki, unpublished work). In addition, different mixed micellar systems at different detergent/phospholipid ratios probably have different equilibration times for achieving (quasi) thermodynamic equilibrium. Also, different detergents may have different 'fusogenic abilities'; for example, the tendency of sodium cholate/lecithin mixed micelles with negatively charged edges to fuse is probably smaller than that for nonionic detergent/lecithin micelles (Y. Nozaki & D. D. Lasic, unpublished work). All these factors probably result in the different size distributions of vesicles formed. In particular, in the case when vesicle preparation rate is comparable to or shorter than the equilibration times of mixed micellar systems, the size of the vesicles produced may depend on the rate of detergent removal (i.e. kinetic effect: Lasic, 1985, 1987). In general, slower detergent depletion rates produce larger vesicles because micelle fusion is not an instantaneous process (Fischer & Lasic, 1984; D. D. Lasic & Y. Nozaki, unpublished work). However, one has to be very careful in interpreting the influence of starting detergent/phospholipid ratio, rate of detergent removal, etc., on vesicle size distribution. This is especially true for dilution techniques where microscopic processes may occur practically independently of macroscopic parameters, such as dilution rate and where, in addition, the remaining detergent may catalyse vesicle fusion (Fischer & Lasic, 1984; Almog *et al.*, 1986; Jiskoot *et al.*, 1986). In the limit of thermodynamic equilibrium (slow removal) the size of the final vesicle preparation depends mainly on $\gamma_{eff.}$ and k_c (with both being a function of temperature and the nature and concentration of remaining detergent). In nonequilibrium conditions smaller (and not necessarily reproducible) vesicles are formed. In such a case size may depend on experimental parameters such as mixing rate, rheology, shape of vessel etc. In the extreme case of forming vesicles very far from equilibrium small SUV and/or phospholipid precipitates result (Fischer & Lasic, 1984; Lasic, 1987).

From these simple considerations two limiting pathways of vesicle growth may be depicted. If $\gamma_{eff.}$ is small (effective shielding of boundaries), $E_b < E_c$ for large values of r and large disk-like micelles can grow and large vesicles result. For large values of $\gamma_{eff.}$ the growing disk-like micelle would be forming the surface of a sphere $R \gtrsim r_c/2$. In reality, however, we can see from Figs. 2(*a*) and 2(*b*) that, according to this simple model, mixed micelles grow in a flat-like manner. In the case of large values of $\gamma_{eff.}$ they start to bend only in the last stages (Fig. 2*a*). In the case of effective shielding (small $\gamma_{eff.}$, Fig. 2*b*) they grow in a flat-like shape up to the final oscillation because $E_b(n)$ for the flat micelle with n molecules is always lower than $E_e(n)$ for the curved micelle $[E_e(n) = E_b(n) \cdot \rho + E_c(n); \quad \rho = \sqrt{(1 - r^2/4R^2)}]$. When $E_b > 8\pi k_c$, the abundant thermal energy (kT per molecule in the aggregate of n molecules) and/or some other possible excitations force the large (oscillating) micelles over the energy barrier for curving ($8\pi k_c$) and the system stabilizes in its lower energy state – that of a closed vesicle. If two unilamellar vesicles (R) fuse into larger unilamellar vesicle ($R\sqrt{2}$) their total E_c is halved. However, this process is entropically unfavoured with contributions (from translational, rotational, vibrational, configurational, conformational and mixing entropy) yet to be evaluated.

Some chaotropic anions can also shield edges of the phospholipid micelles, and a similar method for the vesicle preparation was published (Oku & MacDonald, 1983).

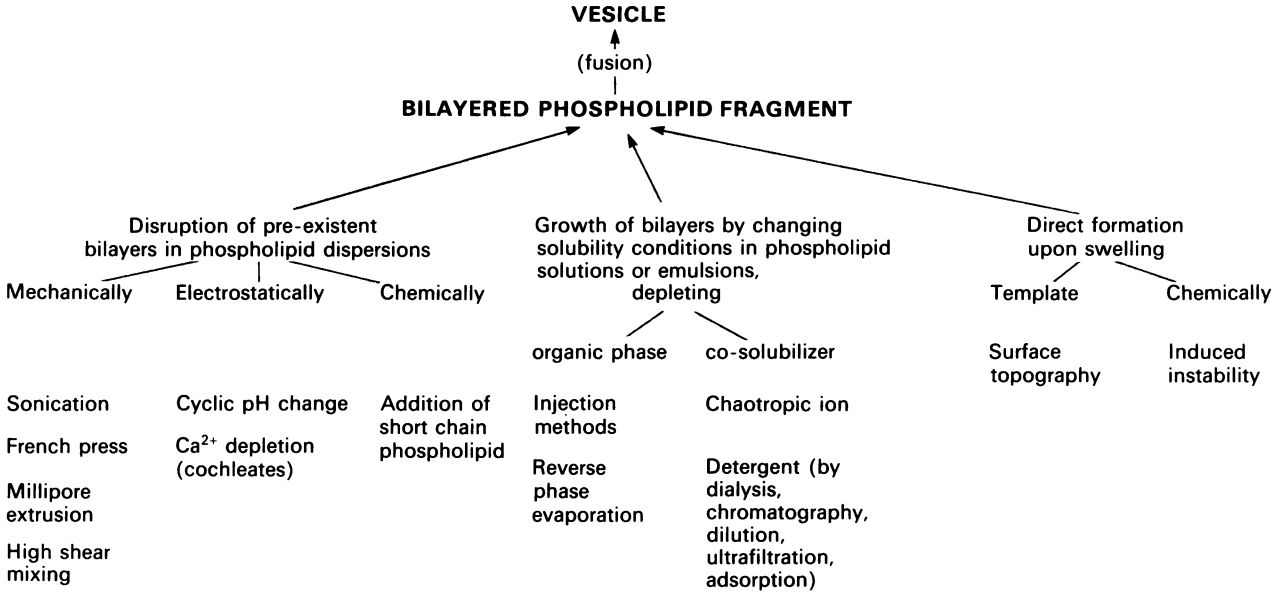
Generalization of this model to the other vesicle preparation methods

Using the concepts of vesicle formation as described above (in particular the disk-like micelle as an intermediate structure in the vesicle formation process) one can easily envisage the geometrical transformations yielding vesicular structure in vesicle preparations by other techniques. Because the other preparation techniques do not involve the use of detergent the micelles are short-lived and resemble a flake of phospholipid bilayer. From here on these will be called bilayered phospholipid fragments (BPF). Different preparation methods produce these intermediate structures in different ways. When a dispersion of MLVs is sonicated or forced through a French press the pre-existent bilayers are torn apart into BPFs which are unstable, due to their exposed edges. By closing upon themselves (if their $r < r_c$ they have to fuse first) they eliminate this unfavourable interaction and vesicles are formed. BPFs can also be formed by the change of solubility conditions: in the injection methods the phospholipid which was dissolved in the organic phase precipitates at the phase boundary water/gas bubble and while bubble rises the crystallized (precipitated) bilayers peel off and form BPFs which vesiculate. The ethanol injection method, however, resembles also the de-emulsification techniques, such as reverse phase evaporation (see below). Bilayers are also preformed in the cochleate technique and the cyclic titration method. The removal of Ca^{2+} ions forces the cochleate cylinder to open; the BPF so formed seals into LUV. In the cyclic titration the lamellae of the dispersed ionic phospholipid ionize, repel one another and BPFs are formed as well.

The mechanism of vesicle formation by reverse phase evaporation technique has already been discussed by Szoka & Papahadjopoulos (1978). For the techniques which start from reverse phases (water-in-oil emulsions), the existence of BPF is less obvious. The inverse micelles with phospholipid located on the phase boundary organic solvent/water transform to vesicles upon removal of the organic phase. These micelles grow in size, transform into a gel formed of a continuous framework of curved bilayered surfaces which upon addition of water disconnect and close upon themselves or in some cases break down into curved BPFs which immediately vesiculate. In any case, a planar bilayer is a necessary intermediate structure in the transition reverse micelle–vesicle.

If phospholipid is deposited on surfaces with special surface topography, BPFs of different sizes can be formed already in the crystalline state. Upon hydration they peel off and form vesicles. The size is controlled by a topography of the template surface while the formation of multilamellar structures is prevented by inducing surface charge on the bilayers (Lasic, 1988; Lasic *et al.*, 1987, 1988).

In the mixtures of short chain (Gabriel & Roberts, 1984; or lysolecithin, Hauser, 1987) and long chain



Scheme 3. Classification of different vesicle preparation methods into a unifying scheme according to the proposed model of vesicle formation

phospholipids which vesiculate spontaneously, a somehow different mechanism was proposed (Gabriel & Roberts, 1984). According to that model, short chain phospholipid, when added to the suspension of MLVs, dissolve in the outer monolayer, induce curvature and bud off vesicles. However, this explanation fails when short and long chain phospholipid are codeposited from the organic phase. In the light of the 'BPF model' the short chain phospholipid molecules simply destabilize large bilayered sheets by dissolving in them. These bilayers break down, short chain phospholipids probably shield edges, but the system nevertheless minimizes its E_c by curving and vesiculation. By coprecipitating short chain and long chain phospholipid the unstable BPF are formed already upon swelling.

Unification of vesicle preparation methods according to the 'BPF model'

The introduction of BPF as a common intermediate structure in the vesicle formation process allows the unification of all the preparation techniques into a logical scheme. The different preparation methods, which were briefly introduced and discussed in the preceding sections, can be divided into (*a*) methods where pre-existent bilayers are via BPFs rearranged into SUVs or LUVs and (*b*) methods where BPFs are made by changing solubility conditions during the preparation procedure. Recently, BPFs were prepared directly (*c*). Scheme 3 shows this unification while Scheme 2 illustrates these transformations geometrically. It stands to reason that on the basis of Schemes 2 and 3 many new techniques for vesicle formation may be proposed.

Dehydration–rehydration vesicles (Kirby & Gregoriadis, 1984) and freeze-thawing vesicles are not included into the scheme because these methods start from SUV/LUV solutions. The first one is especially useful for efficient encapsulation requirements. This method is a type of oil-in-water analogue of the reverse phase evaporation procedure in that water-soluble molecules are forced to be encapsulated. One can also speculate that an analogous technique could be performed where a water soluble drug is added to SUV/LUV/MLV solution externally and, instead of being dried and rehydrated, the solution/dispersion is emulsified with organic solvent and upon removal of the solvent MLVs with a high yield of drug entrapment are produced. Again, understanding these principles and including the structure preservation effect of sugars a whole variety of new techniques can be envisioned.

ALTERNATIVE MODELS

Despite widespread studies of vesicles only a few articles have tackled the mechanism of their formation. The energy considerations of open disks were qualitatively discussed by Ferguson & Brown (1968). Finer *et al.* (1972) proposed a mechanism of vesicle formation by sonication suggesting that ultrasound induces collisions between vesicles which in turn disrupt and the resulting short-lived bilayer fragments or other forms of small aggregates rearrange into vesicles. The energy of a bilayered fragment was described by Helfrich (1974). Zwizinski & Wickner (1977) tried to explain the formation of asymmetric lipoprotein vesicles. However, their model of inverse-normal phospholipid micelles (vesicles without internal cavity) fusing into SUV is very vague and unrealistic. The model of reverse phase evaporation vesicle formation (Szoka & Papahadjopoulos, 1978) cannot be generalized to vesicle preparation procedures which start from normal (oil-in-water) phases.

After a model of vesicle formation by detergent depletion was proposed (Lasic, 1982*a*,*b*) several groups tried to confirm this model in which a disk-like micelle is predicted as an intermediate structure in the vesicle formation process (Schurtenberger *et al.*, 1984; Fromherz & Ruppel, 1985; Almog *et al.*, 1986; Cornell *et al.*, 1986) (see the next section). Later this model was also generalized to explain other preparation techniques (Lasic, 1983, 1987) and the mechanisms of the processes involved

in the vesicle formation by all these methods were qualitatively described.

Several other researchers, however, believe that energy and entropy loss in the formation of BPF is too large. They prefer a model where a gradual stripping down of the multilamellar aggregates occurs in line with the comment made by Evans (1986) that vesicles form by 'budding off' of highly curved sections of larger lipid sheets. Unfortunately none of these models is published except for a qualitative picture of vesicle formation by addition of short-chain phospholipid (Gabriel & Roberts, 1984). Wrigglesworth *et al.* (1987) also described that by detergent depletion large multibilayered sheets are formed which on agitation fragment into SUVs (see below).

The 'BPF' model versus the 'Budding Off' model

The term 'BPF model' is used to describe the model where a bilayered phospholipid fragment (BPF) is an intermediate structure in the vesicle formation process while the 'budding off' model could be described as a reverse fusion model, where large bilayered sheet(s) or MLV 'budd(s) off' SUVs or LUVs. This process is energy requiring because of high curvatures involved and the formation of bilayer defects in the separation step. Also, a very homogeneous size distribution of sonicated vesicles is not easy to encompass with such a model. One can still imagine that resonant vibrations would create bumps ('echinocytic vesicles') on large MLVs or phospholipid sheets which would 'budd off' producing smaller vesicles. The final stages, however, where a vesicle with diameter of ~ 28 nm would have to 'budd off' a vesicle with diameter 20 nm is more difficult to imagine. Also, the gradual decrease in size of MLVs, with formation of small MLVs, LOVs, LUVs, SOVs with intermediate diameters, which would be expected by the 'budding off' model was experimentally not observed. It was shown that MLVs transform directly into SUVs and that no population with intermediate size distribution is present (Finer *et al.*, 1972). Against such a model is also the fact that in several preparation methods large multilamellar structures probably do not occur in the vesicle formation process.

However, such a model was proposed for the vesicle formation by adding short chain phospholipid to MLVs (Gabriel & Roberts, 1984). If this model can describe this process it probably fails to explain spontaneous formation of vesicles from codeposited short chain/long chain phospholipids. More realistic is the assumption that phospholipid bilayers are destabilized by large amounts of the dissolved short chain phospholipid within the bilayers and break down into BPFs, with short chain phospholipid possibly shielding the exposed rims. (This is analogous to the formation of mixed micelles upon addition of detergent to MLVs or LUVs/SUVs. However, these micelles are presumably more stable.) These structures themselves are unstable (large $\gamma_{eff.}$) and vesiculate.

Nevertheless, the initial stage in the formation of BPF can probably occur via the convex curving of the bilayer due to the larger surface of the outer monolayer in which short chain phospholipid is preferentially dissolving. The same is probably true for the mechanism of BPFs formation in the cyclic titration method. The outer polar heads ionize first, increasing their polar head group areas. As a consequence a blister is formed through which water penetrates, which ionizes the inner polar heads causing the BPF to peel off. The two methods indicate that curvature may be induced either by a change in the polar head region or in the non-polar part of the bilayer. Of course one can add amphiphiles which would create changes in both regions at the same time. One can therefore say that within these two methods the initial stage of vesicle formation is the budding off of curved BPFs rather than vesicles.

A 'budding off' model appears capable of explaining successfully the formation of LOVs, and smaller MLVs by the polycarbonate filter extrusion method. Here the large MLVs are forced through smaller holes. Presumably some of the membrane can stretch through the hole and then the applied pressure causes the membrane to burst and it immediately seals off while passing through the hole. The correlation between size of the particles and size of holes supports such a mechanism. However, such budding off can be termed also as a creation of previously curved BPFs.

Vesicles are formed probably in a similar way also in a living cell where cylindrical vesicles of Golgi apparatus pinch off vesicles (with diameter 40–80 nm) at their ends. The driving force for this process is likely to be the action of the cytoskeleton. The manner in which the membranes of the first living organisms were formed, however, still remains a question. Perhaps from the discussion above new models could be proposed.

EXPERIMENTAL EVIDENCE

A direct experimental follow-up in most of the preparation techniques is practically impossible. The transition structures in these processes are formed and maintained by external influences (acoustic, mechanical energies, electrostatic shocks, concentration gradients, change in solubility conditions, evaporation, demulsification) which generally do not allow any simultaneous monitoring. These transition structures are highly unstable and when the preparation procedure can be interrupted for an analysis the results obtained are ambiguous. Probably the detergent-depletion method is currently the only one which allows some realistic experimental monitoring without drastic interferences within the system. However, one must keep in mind also that mixed micelles, especially at higher phospholipid/detergent ratios are inherently unstable. Currently, when even the shapes and sizes of simple detergent micelles are controversial (see, for instance, Lasic & Hauser, 1985, and refs. 1–16 therein), the experimental goal of a direct observation of BPF or large disk-like micelle is still problematic.

We have tried to observe mixed micelles of sodium cholate and egg yolk lecithin and octylglucoside/lecithin at different detergent/phospholipid ratios by negative staining and freeze fracture electron microscopy (D. D. Lasic, Y. Nozaki, J. Beal & J. Costello, unpublished work). Both techniques yielded some qualitative picture of flat, disk-like flakes increasing in size with decreasing detergent/phospholipid ratio. The use of octylglucoside instead of sodium cholate resulted in larger structures. E.s.r. lineshapes of fatty acid and lecithin spin labels dissolved in cholate/lecithin and octylglucoside/lecithin mixed micelles samples during detergent removal, or prepared and equilibrated at fixed values of detergent/phospholipid, were consistent with a bilayered structure

(increasing size with lowering detergent/phospholipid ratio or increasing time of dialysis) while n.m.r. data of the same samples showed that all polar heads were in contact with the added paramagnetic shift ions. Also, quasi-elastic light scattering data of octylglucoside/lecithin micelles showed increasing size with decreasing detergent/phospholipid ratio. The sizes of these micelles were larger than those of bile salt/lecithin micelles at similar detergent/phospholipid ratios (H. Hauser, P. Schurtenberger & D. D. Lasic, unpublished work).

All these results are consistent with disk-like micellar structures but are not, unfortunately, absolutely unambiguous due to possible artifacts during sample preparation, instability of the samples and assumptions made in mathematical modelling.

It should be noted that a disk-like cholate/lecithin micelle was proposed already by Small *et al.* (1969). The improved model of a disk-like micelle with detergent distributed predominantly at the disk edges was presented in 1980 (Mazer *et al.*, 1980) and supported by the electron microscopy and light scattering measurements. Neutron scattering experiments of mixed micelles in a diskotic nematic lyotropic liquid crystal showed that two surfactants with different molecular geometry segregate within the micelle. One is predominantly located in the central core while the other is located mainly in the semitoroidal rim of the micelle (Hendrikx *et al.*, 1983). Electron microscopy of sonicated samples of cholate/lecithin (1:1) showed stacks of lamellae (Fromherz & Ruppel, 1985), with stacks being artifacts of the negative staining procedure.

Several other authors were also interested in the mechanism of vesicle formation by detergent depletion and their results are consistent with the scheme proposed by Lasic (Schurtenberger *et al.*, 1984; Almog *et al.*, 1986; Cornell *et al.*, 1986). A slightly different model was proposed by Wrigglesworth *et al.* (1987). They conclude that vesiculation of the detergent/phospholipid system by detergent depletion follows a two-step mechanism. In the first step mixed micelles grow by fusion into large bilayered aggregates which later, upon mechanical agitation, fragment into SUVs. Their electron microscopy and gel chromatography data show qualitatively that the growing disk-like micelle is an intermediate structure in the vesicle formation process. However, it is less likely that these are also large aggregates of bilayers and that the degree of mechanical stress during the final bilayered sheet–vesicle transition would affect the final vesicle size. When a phospholipid system misses the energy trap of SUV (and possibly also the correct chemical kinetics), thermodynamically more stable MLVs or phospholipid precipitates are formed. These aggregates require more energy than is provided in the dialysis cell/bag or in the column in order to be transformed into SUV (sonication, French press). Also the formation of large sheets would require more energy than the formation of smaller ones and their fragmentation is yet another energy-consuming step. I do not see any reason why the system would have to go through two additional energy-requiring steps if they can be easily avoided by direct closure of smaller disk-like micelles. The observation of large aggregates is probably due to the inherent instability of the mixed micelles at lower detergent/phospholipid ratios to which methods such as negative staining electron microscopy and gel chromatography are especially vulnerable.

The reversible process, i.e. monitoring the dissolution of preformed vesicles with detergents (or chaotropic ions), can also be studied. The dissolving of detergent in vesicles destabilizes them, causing them to open and disintegrate into mixed micelles. The process probably follows the same sequence of intermediate structures with possible hysteresis with respect to the detergent/phospholipid ratio. Again, however, the possible heterogeneity (coexistence of phospholipid-rich/detergent-poor, and vice versa, structures) and instability of the structures make analyses difficult.

PROTEOLIPOSOMES

Vesicles containing proteins (or other macromolecules) within their bilayer (proteoliposomes) are probably formed by the same mechanism (Lasic, 1982, 1983; Casey, 1984). The inclusion of macromolecules within the bilayer may change the k_c of the membrane and in some cases possibly also $\gamma_{eff.}$ (Reijngoud & Phillips, 1984). These two factors probably result in a slightly different size distribution.

This model of vesicle formation predicts random incorporation of proteins. Only in the case of SUV and proteins with very different hydrophilic parts would the unfavourable steric, hydration and possible electrostatic interactions in the small internal cavity result in the orientation of macromolecules reconstituted in the membrane.

CONCLUSION

In this article a model of vesicle formation was discussed. A bilayered phospholipid fragment of the bilayer (BPF) was defined as the intermediate structure in the vesicle formation process. Using this structure all the vesicle preparation procedures were qualitatively described and unified into a logical scheme. Some other possible models were also mentioned.

It is hoped that the model presented will: (i) increase our knowledge of the vesicle formation process and reconstitution of proteins, (ii) shed some light on the micro- and macro-scopic parameters which affect vesicle preparation, (iii) inspire new ideas for designing new, simpler, quicker and less harmful vesicle preparation methods, (iv) remove some misunderstanding and misconcepts in this field, (v) reduce the confusion and disorder in describing, correlating and reviewing the various preparation methods, and (vi) inspire some new experiments to follow up the vesiculation process.

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